The role of chemocommunication at prenatal stages: the rabbit as a model

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INTRODUCTION

All mammalians have evolved behavioral and sensorial methods which guarantee their survival, especially during the first hours of life. In this regard, chemosensory olfactory systems play a pivotal role. Comprising the main (MOS) and accessory olfactory systems (AOS) (Mucignat-Caretta et al., 2012), the former is mainly related to associative behavior whereas the latter is known to mediate innate responses (Marom et al., 2019). In rabbits, the maternal mammary pheromone (2MB2) releases the nipple-search behavior (Schaal et al., 2003). However, pups do not need to learn postnatally pheromonal cues since pups delivered by caesarean section have already great capability to attach to nipples. Therefore, the responsiveness to the pheromone is likely to be learned prenatally (Hudson, 1985). Despite some studies linking this pheromone to the MOS (Charra et al. 2013; Schneider et al. 2016), it has not yet been determined which receptors are linked to this pheromone and where they are specifically located. Furthermore, all of them were performed at postnatal stages in which associative behavior becomes significantly important. Conversely, the AOS has been extensively considered as the responsible of innate behavior as well as the main system for the detection of pheromonal signals (Holy 2018; Mohrhardt et al., 2018).

Furthermore, Pedersen et al. (1985) have demonstrated neuronal activity through 2DG injection in the accessory but not in the main olfactory system of prenatal rats, which suggests that the AOS may be the pathway by which fetal rats detect the odor quality in uterus -odors dissolved in a liquid medium-. Hence, only after birth this function can turn into the detection of airborne odors from the MOS.

In rabbits, only recent studies from our group (Fig 1) (Villamayor et al., 2018, 2019) have shown high degree of development and differentiation of the adult rabbit vomeronasal organ (VNO) and accessory olfactory bulb (AOB) respectively. Our main purpose now is to verify the prenatal maturity of the rabbit VNS. The present work aims to provide a morphofunctional description of its development at three prenatal stages (E20, E26 and E30). We have employed microdissection, specific histological staining techniques and a wide range of immunohistochemical and lectin markers.

FIGURE 1:

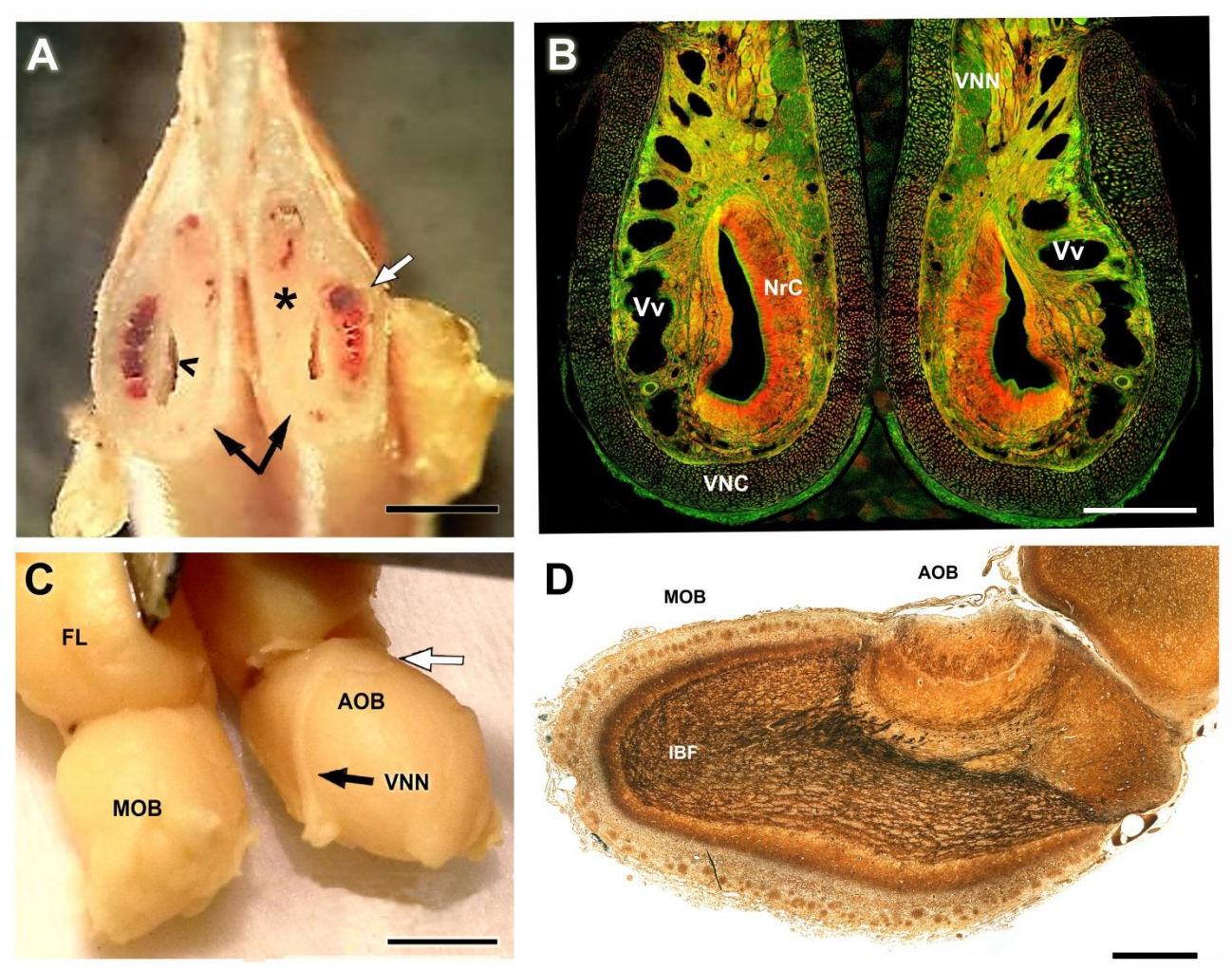

Fig. 1 (A) VNO dissected out, showing its main three components: capsule (black arrows), duct (^) and parenchyma (*) containing wide vascular sinuses (white arrow). (B) Autofluorescence transverse section of the VNO: vomeronasal cartilage (VNC), vomeronasal nerves (VNN), venous sinuses (Vv) and neuroreceptor cells (NrC). (C) Rostro-medial view of the left VNN and AOB. FL, frontal lobe. (D) Bielschowsky-stained sagittal section of the AOB, showing its shape and layers as well as the intrabulbar fibers (IBF) of the MOB. Scale bars: 2 mm (A); 250 µm (B); 2,5 mm (C); 1 mm (D).

MATERIALS & METHODS

We employed the offspring of 9 pregnant females who were in different pregnancy stages: 30 (group 1), 26 (group 2) and 20 days (group 3); 3 animals from each group. We used 3 puppies from each litter, having therefore 9 offspring per group. Mothers were humanely killed under current legislation [Council Regulation (EC) 1099/2009] and the pups were immediately separated in order to study their VNS. Two out of nine of the rabbit heads from the group 1 were dissected fresh for macroscopic studies of the anatomy of the VNS. Due to their little size, the remaining heads were dissected only superficially and immediately processed for their histological study. Thus, the specimens were either fixed by immersion in 10% buffered formalin or kept in Bouin's fixative for 24 h and then transferred to 70% alcohol. After fixation, both the nasal cavities and olfactory bulbs were dissected out to be processed without decalcification. The samples were embedded in paraffin wax and cut into serial transverse sections 3–7 μm thick for examination of the VNO and its nasal cavity topography as well as the AOB (Fig. 1).

The sections were stained with haematoxylin-eosin, Gallego's trichrome (Ortiz-Hidalgo, 2011), periodic acid-Schiff (PAS), Alcian blue and Nissl. Furthermore, an histochemical and immunohistochemical analysis was carried out. We studied 3 lectins: UEA, which recognize the L-fucose moiety of glycoproteins and it is a specific marker of the vomeronasal pathway; BSI-B4, as an excellent specific marker of the VNS in both rats (Salazar & Sanchez-Quinteiro, 1998) and opossums (Shapiro et al.1995); and LEA, which is specific for the olfactory system (nervous and glomerular layers). The immunohistochemistry studies required the blocking of both endogenous peroxidase activity and non-specific binding with hydrogen peroxide and BSA, respectively. Then, the primary antibody was added (Table 1) and incubated overnight. The next day, the cells were incubated for 1.5 h in the corresponding biotinylated secondary antibody and for 1.5 h at room temperature in Vectastain ABC reagent (ABC, Vector Laboratories, Burlingame, CA, USA). All procedures followed the guidelines for housing and handling provided by the Bioethical Committee of the University of Santiago de Compostela, and conformed to European legislation (EU directive 2010/63/EU) and Spanish legislation (RD 53/2013).

RESULTS

FIGURE 2:

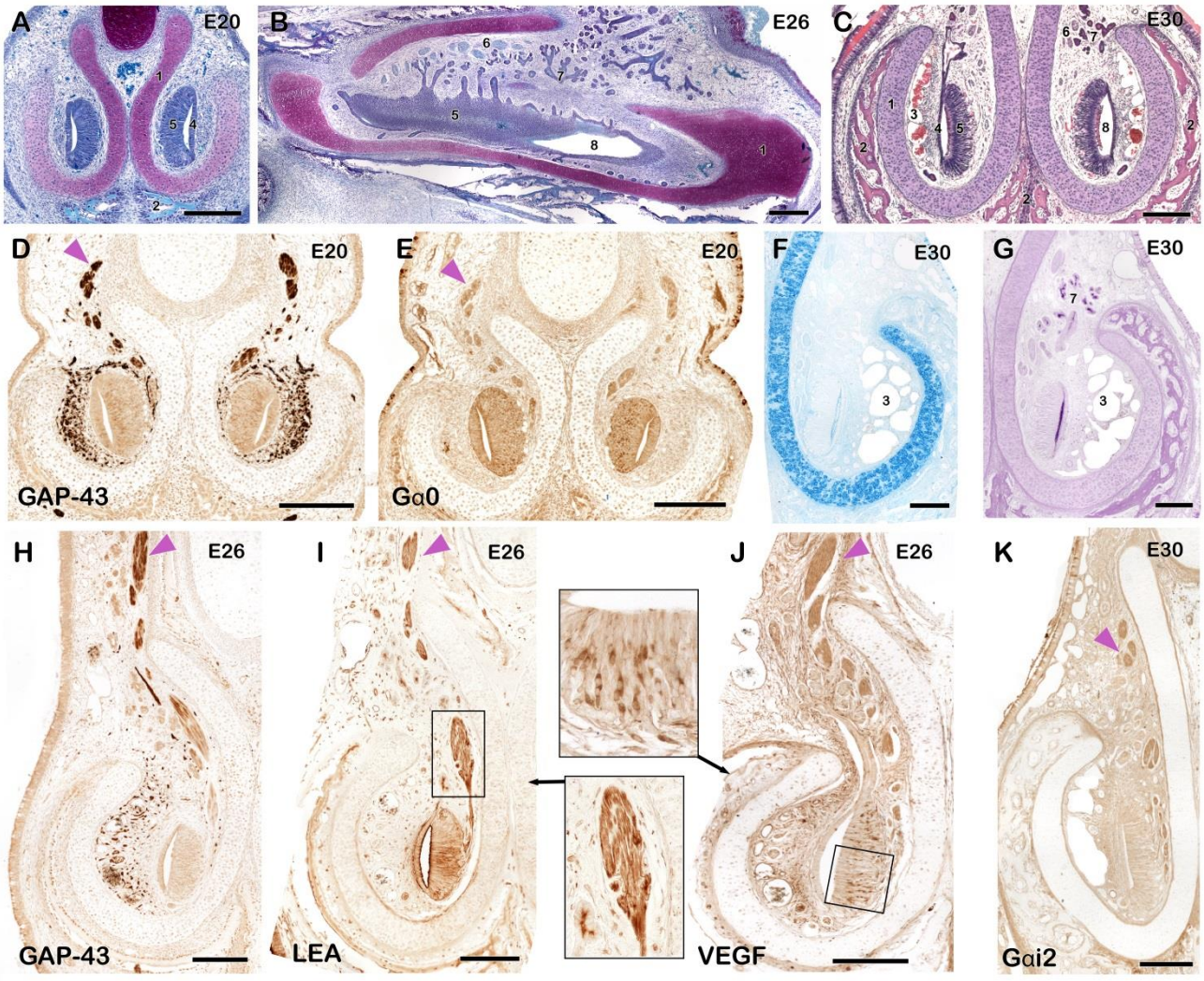

Fig. 2 Histological and immunohistochemical study of the rabbit OVN at prenatal stages. A-C: Histological sections stained with Gallego Trichromic at E20 (A) and E26 (B, sagittal section) and with H&E at E30 (C). Cartilage (1), bone (2), venous sinuses (3), respiratory epithelium (4), sensory epithelium (5), nerves (6), glands (7), duct (8). F-G: Histological sections of the VNO at E30 stained with Alcian blue (F) and PAS (G). D-E & H-K: Immunohistochemical labelling against different proteins at different prenatal stages. E20: GAP 43 (D) shows neuronal activity at both sensorial and autonomous innervation and Gα0 (E) labels the vomeronasal pathway. E26: GAP 43 (H) shows the same pattern but less strong than E20, and LEA (I) labels the typical vomeronasal pathway. A higher magnification of the vomeronasal nerve is shown in the inset. VEGF (J) stains individual neurons migrating along the lamina propria of the sensory epithelium, as it is shown in the inset. E30: Gαi2 (K) stains the vomeronasal nerves. Pink arrowheads: vomeronasal nerves. Scale bars: 100 µm (C); 200 µm (A, D-E, H-K); 100 µm (C); 300 µm (B, F, G).

***FIGURE* 3:**

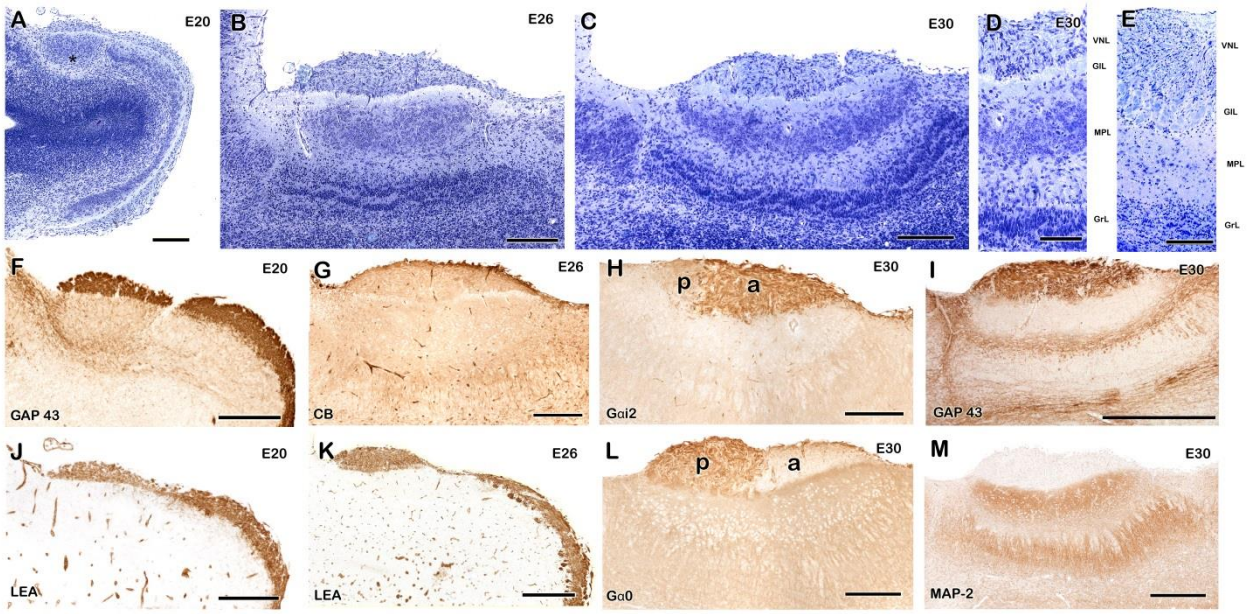

Fig. 3 (A-E) Nissl-stained sections of the AOB at different ages. (A) E20, within the OB (B) E26 and (C) E30. (D-E) They show the different layers of the AOB in E30 (D) which are still under development without a defined glomerular layer but with a profuse mitral cell layer, and in the adult (E), which have established boundaries between layers but less mitral cells compared to E30. VNL, Vomeronasal nerve layer; GlL, Glomerular layer; MPL, Mitral-plexiform layer; GrL, Granule cells layer. (F-M) Immunohistochemical labelling against different proteins at different prenatal stages. E20: Anti-GAP-43 strongly labels the VNL in both AOB and OB (F); LEA labels both the vomeronasal axons and glomeruli in the whole AOB and OB (J). E26: anti-CB is noticeable for first time (G) and LEA labelling is stronger and the OB shows small glomeruli (K); E30: Anti-Gαi2 stains the anterior (a) VNL/GlL of the AOB (H) whereas anti-Gαo (p) stains the posterior half of the AOB (L); anti-GAP 43 strongly labels the superficial and granular layers at this age in the same way as in adults (I); anti MAP-2 stains strongly the plexiform and granular layers (M). Scale bars: 200 µm (A-C, F-J, L-M); 100 µm (D-E); 400 µm (K).

DISCUSSION

Although rabbits have become a model within the study of mammals chemocommunication (Schaal et al., 2003, Schneider et al., 2018), there is a lack of morphological information about the structural features of the vomeronasal system at prenatal stages. Only recently, ALomaisi et al. (2019) have studied the morphology of the OVN at different prenatal stages. They have determined its first appearance as well as its developmental changes. However, they did not address the functionality and maturity of the OVN and what is more, its correlation with the accessory olfactory bulb, its first integrative centre within the nervous system.

Our observations confirm that the rabbit possesses a highly developed VNO, which grows gradually in size and differentiation from at least E20 to E30. In E20 the OVN clearly shows two different types of epithelium. The medial wall is lined along the vomeronasal duct with a thick layer of neurosensory epithelium (receptor), whereas the lateral wall is covered by a thinner non-sensory ciliated epithelium (respiratory), following therefore the same pattern as in the adult (Villamayor et al., 2018) and in the majority of species (Halpern & Martinez-Marcos, 2003). At this age, the OVN is also provided with a double envelope which is cartilaginous internally is bony externally. Regarding the glandular component, our results show a quite incipient staining at E20 for neutral mucopolysaccharides with PAS but contrarily not staining at all for acid mucopolysaccharides with Alcian blue (Ab). It seems to follow the same pattern as in adults (Villamayor et al., 2019) and other mammals such as cats (Salazar et al. 1996) or horses (Lee et al. 2016). Immunohistochemical labeling with GAP 43 or Gα0 shows the presence of growing nerves –both sensorial and autonomic– at E20.

At E26 highly developed venous sinuses stand out. This observation indicates the crucial role played in this species by the pumping mechanism that introduces chemical signals into the vomeronasal duct. The functional properties of the organ at this age are also confirmed by the presence of a well-developed neuroepithelium and profuse glandular tissue that is positive for neutral mucopolysaccharides. At E30 the VNO increase in size and development. In addition, some immunostaining as anti-VEGF or anti-CB appear to be stronger than at earlier stages.

Regarding the accessory olfactory bulb (AOB), it can be already differentiated from the olfactory bulb (OB) at E20. Nevertheless, both OB and AOB are very primitive at this age and there is not clear difference between different cell types. At E26 and then E30 the previous indistinguishable cell types turn into different subtypes, forming therefore arranged AOB layers, but still with lack of boundaries between them. Despite the lack of differentiated borders between layers even at E30, there is already some immunohistochemical

labelling at E20, which suggests that the AOB is already mature at this stage. Comparing to the adult rabbit, this latter has a full developed AOB in terms of its size, topography, cell density, and thickness as well as sharpness of boundaries among AOB layers (Villamayor et al., 2019), which are surely established at the early postnatal stages.

In conclusion, the development of the VNS can be traced from at least E20, and it steadily increases until E30. The rabbit VNS is already functional at prenatal stages and it gains maturity from at least E26, when glands are functional and most immunohistochemical markers provide strong staining. The maturity of the VNS suggests that the bond between female-offspring is already established prenatally. In this regard, the pheromone 2MB2 might reach the OVN in uterus, triggering innate behavior which will allow pups to learn the sucking behavior once they born.

Further studies regarding the gene expression (for instance through RNAseq) of the VNS at prenatal stages are required in order to assure its functionality and prove which receptors are able to recognize the 2MB2 pheromone among others.

BIBLIOGRAPY

ALomaisi S, El-Ghazali H, Nosseur H, El-dein Ahmed S, Konsowa M (2019) Prenatal development of the vomeronasal organ in rabbit. *Slovenski Veterinarski Zbornik* 56 (22): 623-32

Charra R, Datiche F, Gigot V, Schaal B, Coureaud G (2013) Pheromone-induced odor learning modifies Fos expression in the newborn rabbit brain. *Behav Brain Res*. 15 (237): 129-40

Holy TE (2018) The Accessory Olfactory System: Innately Specialized or Microcosm of Mammalian Circuitry? *Annu Rev Neurosci.* 41: 501-525

Hudson R (1985) Do newborn rabbits learn the odor stimuli releasing nipple-search behavior? *Developmental Psychobiology* 18 (6), 575-585

Lee KH, Park C, Kim J, et al. (2016) Histological and lectin histochemical studies of the vomeronasal organ of horses. *Tissue Cell* 48, 361–369.

Marom K, Horesh N, Abu-Snieneh A, Dafni A, Paul R, Fleck D, Spehr M, Ben-Shaul Y (2019) The vomeronasal system can learn novel stimulus response pairings. *Cell Reports* 27 (3): 676-684

Meredith M (1991) Sensory processing in the main and accessory olfactory systems: comparisons and contrasts. *J Steroid Biochem Mol Biol.* 39 (4B): 601-14

Mohrhardt J, Nagel M, Fleck D, Ben-Shaul Y, Spehr M (2018) Signal Detection and Coding in the Accesory Olfactory Bulb. Chem Senses 43 (9): 667-695

Mucignat-Caretta C, Redaelli M, Caretta A (2012). One nose, one brain: contribution of the main and accessory olfactory system to chemosensation. *Frontiers in Neuroanatomy* 6: 46.

Pedersen PE, Stewart WB, Greer CA, Shepherd GM. Evidence for olfactory function in utero. *Science* 29: 221(4609): 478-80

Schaal B, Coureaud G, Langlois D, et al. (2003) Chemical and behavioural characterization of the rabbit mammary pheromone. *Nature* 424, 68-72.

Schneider NY, Piccin C, Datiche F, Coureaud G (2016) Spontaneous brain processing of the mammary pheromone in rabbit neonates prior to milk intake. *Behav Brain Res.* 15 (313): 191-200

Salazar I, Sanchez-Quinteiro P, Cifuentes JM, et al. (1996) The vomeronasal organ of the cat. *J Anat* 188, 445–454.

Villamayor PR, Cifuentes JM, Fdz.-de-Troconiz P, Sanchez-Quinteiro P (2018) Morphological and immunohistochemical study of the rabbit vomeronasal organ. *J Anat* 233, 814-27

Villamayor PR, Cifuentes JM, Quintela L, Barcia R, Sánchez-Quinteiro P (2019) Structural and Immunohistochemical study of the rabbit accessory olfactory bulb. Submitted to *Brain Structure and Function*